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Single Nucleotide Polymorphisms (SNP)-specific Quantitative Real Time Polymerase Chain Reaction (PCR) Assay for Analyzing Competition and Emergence of the Military Hypersporulating Strains of *Bacillus Atrophaeous* var. *Globigii*

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14. ABSTRACT

The utility of single nucleotide polymorphisms (SNPs) for detecting phenotype-specific subpopulations is increasing with the increased understanding of the role of SNPs in adaptation. Based on the recently identification of the Spo0FH101R mutation as phenotype-defining for the hypersporulating military strains of *B. atrophaeus*, we are developing an assay to detect and quantify hypersporulating military strains of *Bacillus atrophaeus var globigii* (BG) from mixed cultures with ancestral low spore yield *Bacillus atrophaeus* variants using the (C:T) SNP corresponding to the Spo0FH101R mutation. The *B. atrophaeus* congenic pair Detrick-1: Detrick-2 is used for assay validation. The assay is internally calibrated and does not require separate internal-control polymerase chain reaction (PCR), making it high-throughput compatible. Assay specificity 1:1000 is achieved, meaning one spore/cell of Detrick-2 bacteria is detected in the background of 1000 Detrick-1 spores/cells. A novel approach is proposed for analyzing competition experiments based on the relative PCR quantification method and is tested in a hypothetical experiment of emergence of either Detrick-1 or Detrick-2 using mixed bacterial cultures with varying strain frequencies. Strain emergence, characterized by the rate of change in frequency, is calculated from PCR data using our approach. The fitness parameters calculated from the experiments are in good agreement with the theoretical fitness parameter, thus validating the approach's utility.

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1. Objective

Identification and detection of genomic signatures has become important tool in studying the development of drug resistances by pathogens or the emergence of high morbidity and/or high mortality pathogenic strains. The ability to perform competition experiments using native strains is essential for understanding the conditions for strain emergence in natural environments or the interaction of pathogens with their hosts. This report develops methodology for analyzing bacterial competition experiments or experiments of strain emergence using native strain-specific genomic signatures. The goal is to provide a sensitive and fast method for measuring relative fitness parameters of non-engineered bacterial strains with sufficient selectivity to allow for environmental applications. The proposed methodology has potential applications in forensic genomics, where the contribution of a given mutation to strain fitness is essential for inferring intent in markerless engineered genomes. The relative polymerase chain reaction (PCR) quantification method is chosen as the application platform for the proposed methodology and two historically important military strains of *Bacillus atrophaeus*, Detrick-1 and Detrick-2, are used as an example. Detrick-1 and Detrick-2 are congenic strains with single (C:T) substitution corresponding to the Spo0FH101R mutation. Our accomplishments include the development of quantitative assay for detection of the (C:T) single nucleotide polymorphism in *spo0F*, which is suitable for high-throughput screening of mixed bacterial cultures. The assay has selectivity 1:1000, which means that one hypersporulating bacteria carrying the 101R signature of Spo0F is detected among 1000 bacteria lacking this signature. The utility of the assay is validated in hypothetical competition/emergence experiments for either Detrick-1 or Detrick-2. The assay has been applied successfully in a separate work for studying the effect of Spo0H101R mutation on strain fitness (*1*).

2. Introduction

B. atrophaeus var globigii (BG) is a sporulating soil bacterium used as a common bio-tracer (*2–6*) and simulant (*7–15*). Recently we performed whole genome molecular typing of several BG strains including the strains used by U.S. military as simulants for biological warfare and bioterrorism events (*16*). The whole genome molecular typing of the military strains showed that a single (C:T) substitution of *spo0F* corresponding to Spo0FH101R mutation supports high spore yield (hypersporulating) phenotype. In natural environments, sporulation in wild type bacillus species is commonly induced by starvation (*17*). The decision to commit to either sporulation or competence is a complex process (*18*), where the multicomponent phosphorelay plays a key role (*19*) and where Spo0F is a major regulator for phosphoryl group transfer from several histidine kinases to Spo0B (*20*) and then to the transcription regulator Spo0A (*21*), which

regulates the expression of more than 120 genes (*22*). The Spo0F protein of BG is identical to the same protein of *Bacillus subtilis* except for two amino acids. Similar directed mutation at the H101 residue of Spo0F of *B. subtilis* (Spo0FH101A) confers a hypersporulating phenotype (*23*). It is believed that mutations at H101 alter the arrangement of the amino-residues in the β4-α4 recognition loop and affect the phosphorelay performance (*24*). The Spo0FH101A mutation in *B. subtilis* is a human-introduced mutation. In contrast, the mutation at the H101R locus of Spo0F in BG occurred spontaneously. It is known that the high spore yield BG strains were intentionally selected in experiments conducted after the Second World War (*25*). However, it is not known whether these strains became fixed in the experimental cultures under specific conditions where the H101R mutation was beneficiary. The inability to produce a viable *B. subtilis* strain carrying a stable H101R mutation suggests that this mutation might not be beneficiary, in which case the fixation under natural conditions of the hypersporulating strains is unlikely (*16*). However, the inability to generate a viable *B. subtilis* strain carrying the H101R mutation could be species dependent.

Bacteria use diverse strategies for survival in changing environments including genomic alterations (*26–28*), phenotypic switching (*29*), or mobilization of cooperative responses (*30*). Mutations in key pathway regulators are rare events, but they represent an important mechanism for increasing phenotypic diversity (*31*). Recent directed evolution studies combined with whole genome sequencing have shown that relatively small number of mutations in key regulators appearing during the first generations under new selective pressure accounts for most of the gains in fitness, and that subsequent mutations often have diminishing effect on cumulative fitness gains (*32–35*). The acquisition of two independent mutations in Spo0F of BG, where each mutation gives rise to a hypersporulating variant (*16*), is one such example of phenotype-defining mutation in a key pathway regulator. One approach for determining whether a phenotype-defining mutation is beneficiary in specified growth conditions is to compete a strain carrying the mutation of interest with its congenic ancestral strain. The BG pair Detrick-1 and Detrick-2 is a congenic pair, where Detrick-1 is hyposporulating strain considered ancestral for the hypersporulating strain Detrick-2. Detrick-2 has one (C:T) substitution in *spo0F* corresponding to the Spo0FH101R mutation (*16*). To compete the two strains, their frequencies in the competing culture need to be measured. Competition experiments using antibiotic resistant strains have been performed for *B. subtilis* strains obtained from directed evolution experiments (*36, 37*). However, the use of antibiotic resistance could introduce unwanted complications to data analysis because spores are naturally resistant to antibiotics. Therefore, here single nucleotide polymorphisms (SNP)-specific quantitative PCR (qPCR) assay is developed for measuring strain frequencies in mixed cultures, which accounts for the cells in the culture at all stages of their life cycle. The (T:C) SNP corresponding to the H101R mutation is used as a marker for distinguishing between the two strains. SNP detection in mixed samples is commonly performed using either end-point detection methods (*38–40*) or real-time PCR (qPCR) (*41–46*). Here SNP-specific qPCR assay based on the relative quantification method

(*47*) is developed and used for calculation of the rate of change in frequency (*48*) in a hypothetical experiments of emergence of either Detrick-1 or Detrick-2.

3.   Materials and Methods

3.1   Bacterial Cultures

The archival strains Detrick-1 and Detrick-2 of BG were obtained as described elsewhere (*16*). Cells were cultured in corn steep liquor (CSL) (Sigma-Aldrich, St. Louis, MO). The stock CSL contained approximately 50% solids and 50% liquid with dissolved water soluble components (*49*). The water soluble components were used for the preparation of culture media as described elsewhere (*25, 50*). Briefly, the stock CSL was centrifuged at 14,000xg for 15 min. The supernatant was collected and its pH was adjusted to pH 7.0 with sodium hydroxide (NaOH). Finally, the supernatant was cleared by performing one additional centrifugation at 14,000xg for 20 min. The cleared supernatant was used for the preparation of 1.5 wt.% CSL growth media. Alternatively, the cells were cultured in lysogeny broth (LB).

To avoid possible cross-contamination, single colonies exhibiting the spo0F(101H) or spo0F(101R) signatures were chosen. Three-day or older colonies were used for starting new cultures. The cells from a single colony were heat-shocked and grown in 1.5% CSL as planktonic cultures to optical density (OD) between 1.0 and 2.0. The cultures were shaken on a rotary shaker at 250 rpm and 33 °C (MaxQ 4000, Thermo Scientific, Asheville, NC). Then, 0.5-ml samples were taken from the single-species cultures and used to determine the percentage of live cells using Invitrogen's LiveDead BacLight assay (Invitrogen, Carlsbad, CA) and flow cytometry (BD FACSCanto II, BD Biosciences, San Jose, CA). Cultures with 94% or higher percentage of live cells were used for preparing mixed bacterial cultures. Strain frequencies in the mixed cultures were adjusted using OD measurements.

3.2   DNA Isolation

First, 6-ml samples from either single species cultures or mixed cultures were collected and washed with phosphate buffered saline (PBS) to remove cell debris. The samples were washed five additional times with PBS containing 10% dimethyl sulfoxide (DMSO) to remove possible polymerase inhibitors. Genomic DNA was isolated using the PowerSoil kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer protocol with two modifications. One, 10 µl of Proteinase K (Sigma) 10 mg/ml was added to solution C1. The cells were incubated in solution C1 for 10 min at 65 °C prior to beating in a bead beater for 40 s. Second, an additional desalting wash with ethanol was introduced after the wash with solution C5. The quality of the obtained DNA was checked using NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

3.3 Real-time PCR

SNP-specific forward primers corresponding to C:T substitution of *spo0F* were custom designed using the sequence information from the whole genome typing study (*16*). The forward primers were: pSpo0F(101H)-D1-f matching the Detrick-1 DNA strand (5'-TCAAAAGGCTTAGCGAAcT-3') and pSpo0F(101R)-D2-f matching the Detrick-2 DNA strand (5'-TCAAAAGGCTTAGCGAAcC-3'). One mismatch in -1 position was introduced (lower case nucleotide) to improve primer performance. The nucleotide at 3' position corresponded to the (C:T) SNP of the spo0F gene (*16*). One common reverse primer pSpo0F(H101R)-186-rev (5'-GCCCTGATCTTGTTCTCCTC-3') was used to produce 186 bp amplicon. Primers specific for BG and producing 228-bp amplicon for the putative RNA helicase (*16*) were designed and used as internal control primers. They were pBG21115-f (5'-AACCGTCGTAAACCAAGTCG-3') and pBG21115-r (5'- TCCAAAGGTTCTGTCCCATC-3'). Three primer sets were assembled using the above primers: set pD1 comprised of (pSpo0F(101H)-D1-f and pSpo0F(H101R)-186-rev), set pD2 comprised of (pSpo0F(101R)-D2-f and pSpo0F(H101R)-186-rev), and set p21115 comprised of (pBG21115-f and pBG21115-r).

PCR reaction volumes were 10 µl, including 500 µM of each primer, a template (template concentration was 10 ng if not specified), and 5 µl of SsoFast-EvaGreen supermix (BioRad, Hercules CA). PCR amplification and fluorescence detection were carried out on either LightCycler (Roche) or CFX-Connect (BioRad) using the white PCR plates for LightCycler. The thermal cycling for LightCycler included a 5-min initial denaturation at 94 °C, annealing/extension for 40 s at 54 °C, and fluorescence acquisition at the end of each extension for 45 cycles. The thermal cycling for CFX-Connect included a 5-min initial denaturation at 94 °C, step down to 60 °C for 3 s, annealing/extension for 40 s at 54 °C, and fluorescence acquisition at the end of each extension for 45 cycles. Melting curve measurements were performed after the completion of cycling of each real-time PCR reaction. The measured C_T values measured using either the LightCycler or the CFX-Connect systems were comparable.

4. Results

4.1 SNP-specific qPCR Assay for Calculation of Fitness Parameters

In competition experiments, two bacterial species are initially mixed at a desired ratio and then incubated in specified growth conditions for several generations to determine whether one of the species has fitness advantage in the chosen conditions. The species with the faster growth rate is considered to have a fitness advantage, which is characterized by the increase of the species' frequency. The logarithm of the frequency ratio plotted against the generation time is used to calculate the rate of change in frequency. This is similar to the calculation of selection coefficient (*51*) or selection rate constant (*52*) of non-interacting cultures during the exponential growth phase. For density dependent cultures or cultures grown in fluctuating environments,

real time is used instead of generation time. The logarithm of the frequency ratio is calculated from

$$\ln\left(\frac{P_{x(t)}}{P_{y(t)}}\right) = \ln(1 + E)\left(C_{T,y(t)} - C_{T,x(t)}\right),$$ (1)

where $P_x(t)$ is frequency of strain x at time (t), E is the efficiency of the PCR reaction, and $C_{T,x(t)}$ and $C_{T,y(t)}$ are the C_T values for strains x and y, respectively. By convention Detrick-1 is considered to be the x strain and Detrick-2 the y strain.

4.2 Percent Live Cells in the Initial Cultures

The PowerSoil kit was used here for genomic DNA (gDNA) isolation from both cultures containing both vegetative cells and spores. The Invitrogen's Live/Dead assay was used to measure the percentage of live cells in the single-species starting cultures of Detrick-1 and Detrick-2 (figure 1). The left graphs in figure 1 show the measured fluorescence from SYTO9 versus front scattering for cells strained with SYTO9 only. The right graphs in figure 1 show the measured SYTO9 fluorescence after the addition of propidium iodide (PI).
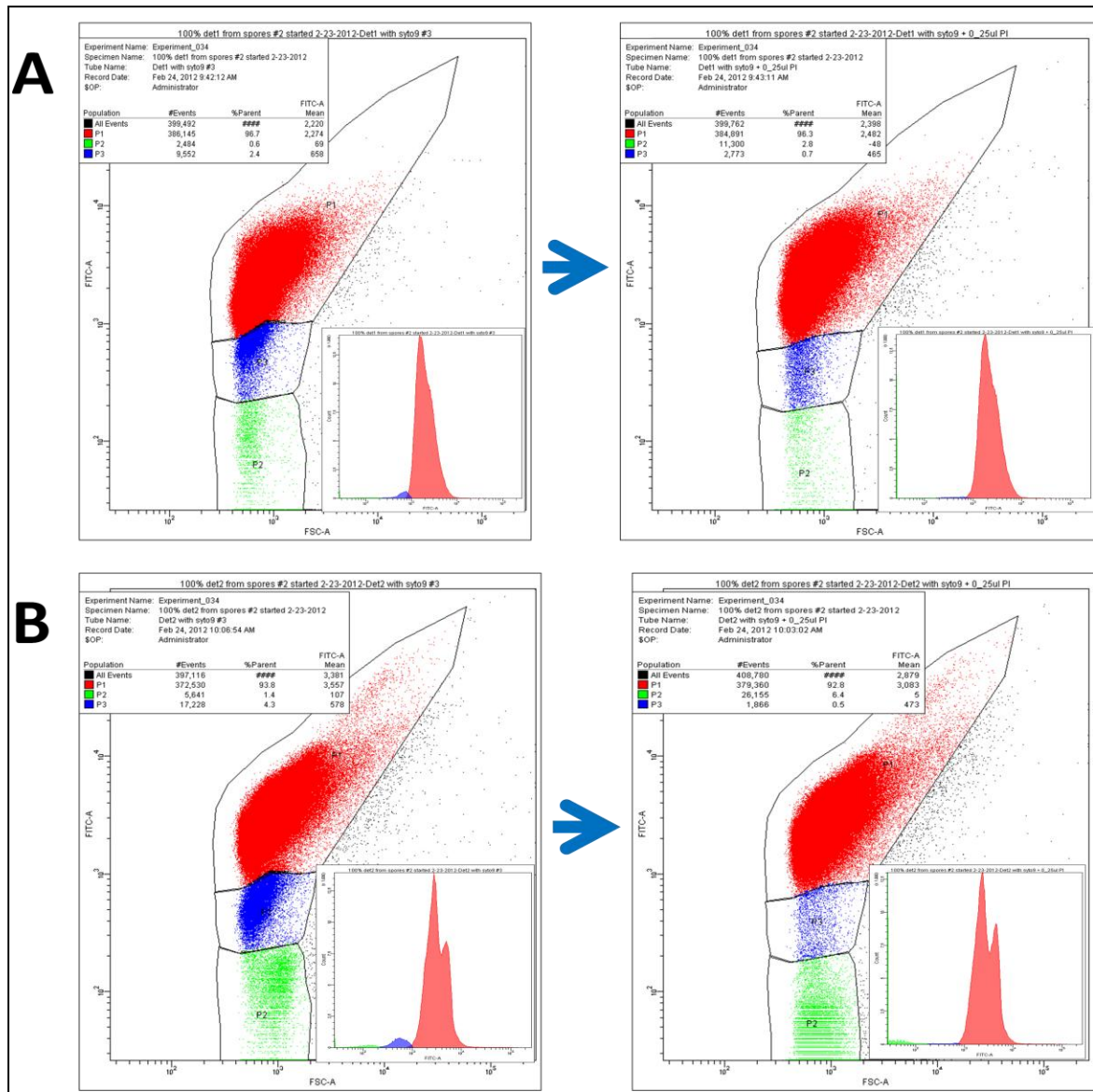
Figure 1. Flow cytometry using the Life/Dead assay (Invitrogen) for single species (a) Detrick-1 and (b) Detrick-2 cultures after 24 h of incubation in 1.5% CSL. The measured fluorescence intensity from SYTO9 is shown in the y-axes and the measured front scattering is shown in the x-axes. The left graphs represents cells stained with SYTO9 only and the right graphs represents cells stained SYTO9 after the addition of PI. The red colored regions correspond to the live cells, the blue colored regions correspond to the dead cells or the cells with compromised membrane pumps, and the green regions correspond to the cell debris or other particles.

SYTO9 is a minor-groove binding fluorophore, which has high membrane permeability and stains both live and dead cells. PI is another minor groove binder, which quenches the fluorescence from SYTO9. PI is pumped out of the cells when the membrane pumps are functional, which prevents the intracellular PI concentration to reach levels sufficient for quenching SYTO9 fluorescence. This effect is used to distinguish between live cells or cells with functional membrane pumps and dead cells or cells with compromised membrane pumps.

Three distinct regions are identified in the graphs shown in figure 1: (a) red-colored region where SYTO9 fluorescence is not affected by the addition of PI identifying the live cell population; (b) blue-colored region where the addition of PI quenches the SYTO9 fluorescence identifying the dead cell population or the cells with compromised membrane pumps; and (c) green-colored region where the SYTO9 fluorescence is at low level before the addition of PI identifying cell debris or other particles. The data in figure 1 show that 96.3% of Detrick-1 cells and 92.8% percent of Detrick-2 cells are live cells, respectively. The dead cell population represents 2.4% of the Detrick-1 population and 4.3% of the Detrick-2 population, respectively. These data show that more Detrick-2 than Detrick-1 cells are either dead cells or have non-functional membrane pumps. This percentage of live cells was taken under consideration during the construction of the mixed cell cultures for the hypothetical emerging experiments discussed below.

4.3 Requirements for Primer Sets Design

The efficacy of the relative quantification PCR method is sensitive to multiple factors including polymerase used, primer quality, reaction conditions, template-dependent bias, amplicon size, etc. The utility of the method is dependent on the ability to minimize the differential effect of these factors when SNP-specific primer sets are used. Figure 2a shows the agarose gel of the products generated in four PCRs. Two of the reactions use gDNA from Detrick-1 as template and two use gDNA from Detrick-2 as template, respectively. Two primer sets are used in each reaction; one is a SNP-specific primer set and the other is the internal control primer set. Two bands are seen in the lanes where the SNP-specific primer sets are matching the used templates. One of the bands corresponds to the SNP-specific primer set and the other corresponds to the internal-control primer set. Only one band is seen in the lanes where the SNP-specific primer sets are mismatching the used template. This band corresponds to the internal-control prime set. No other bands appear on the gel, showing that the designed primer sets are suitable for use in real-time PCR assays.

The SNP-specific primer sets are designed to fulfill the requirement for minimum bias toward the used templates. This is illustrated in figure 2b, where the C_T for the matching templates of the two SNP-specific primer sets are almost equal. The traces from the real-time PCRs using the internal-control primers are also shown as reference. The internal-control primer set is designed choosing a region in the BG genome with specified GC content and size, such that the measured C_T values when the internal-control primer set is used to be similar to the C_T values when the SNP-specific primer sets are used with matching template. This provided for the opportunity to use directly the measured C_T values for the reactions using SNP-specific primer sets in equation 1 without adjustments for template bias.
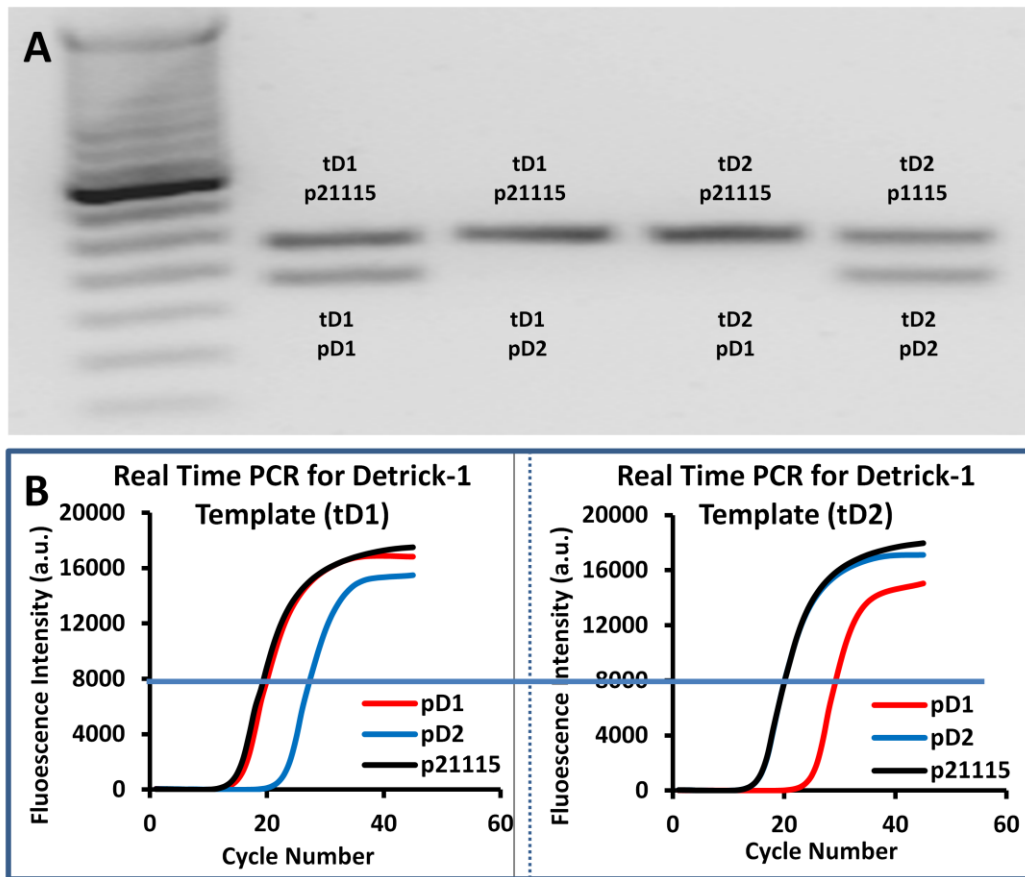
Figure 2. Primer set quality and design requirements. (a) Agarose gel of the products from four real-time PCRs using Detrick-1 and Detrick-2 gDNA as template. The primer sets corresponding to each band are as shown. (b) Traces of measured fluorescence intensity vs. cycle number for real-time PCRs using Detrick-1 and Detrick-2 templates and one of the three primer sets as shown.

4.4   Primer Sets Sensitivity, Specificity, and Selectivity

The performance of SNP-specific primer sets is characterized by sensitivity, specificity, and selectivity. Sensitivity is defined as the minimum number of template molecules detected by the assay. The theoretical limit for PCR sensitivity is one template. This limit is easily approached in the reactions using the internal-control primer set p21115 when the reactions using the SNP-specific primer sets are characterized by lower sensitivity.

Figure 3 shows the results from experiments used to determine assay sensitivity. The reactions with the highest C_T values shown in figure 3 use 10 ng of gDNA per reaction, which corresponds to 4,400,000 copies of gDNA. The template copy-number in each flowing reaction is obtained via (1:9) (v:v) serial dilutions using PCR-clean water. The lowest amount of template per reaction is obtained after six serial dilutions, which corresponds to four templates per reaction. Figure 2a and b shows that when the p21115 primer set is used the dependence of the measured C_T values on the log of template concentration is linear over the full range of serial dilutions

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used. In contrast, when the SNP-specific primer sets are used the above linear dependence is observed only over the range of template copies from 4,000,000 to 40.

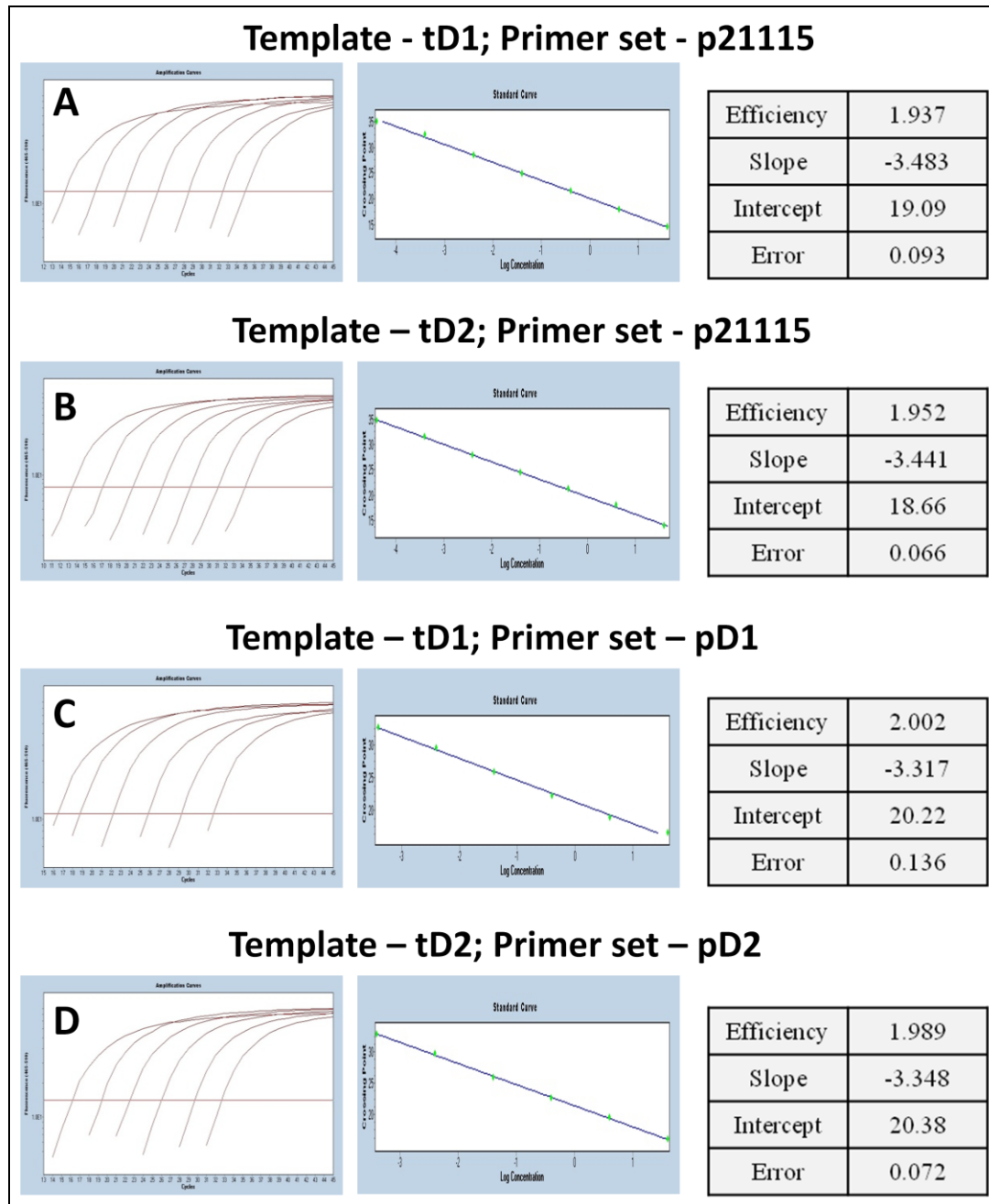Figure 3. Sensitivity of the PCRs using the internal-control primer set and the two SNP-specific primer sets and gDNA as template from either Detrick-1 or Detrick-2. The highest amount of template per PCR is 10 ng, which corresponds to 4,400,000 copies per reaction. The template copy number of each following reaction is obtained via (1:9) (v:v) serial dilutions. The LightCycler480 system was used.

One additional requirement is introduced for all reactions, which is to have efficiency close to 100%. The efficiencies of the reactions using the internal-control primer set were 93.7% for the Detrick-1 template and 95.2% for the Detrick-2 template. The reaction efficiencies increased and approached 100% when the SNP-specific primer sets are used. Therefore, the additional requirement is fulfilled by all primer sets.

Specificity is measured for reactions using SNP-specific primers and is defined as the maximum amount of mismatched template for which the measured C_T values are equal to the C_T values for the control. Figure 4 shows the results of experiments performed in the determination of primer set specificity. The specificity of the pD1 primer set is on the order of 4,400 copies and the specificity of the pD2 primer set is on the order of 2,200 copies or less.
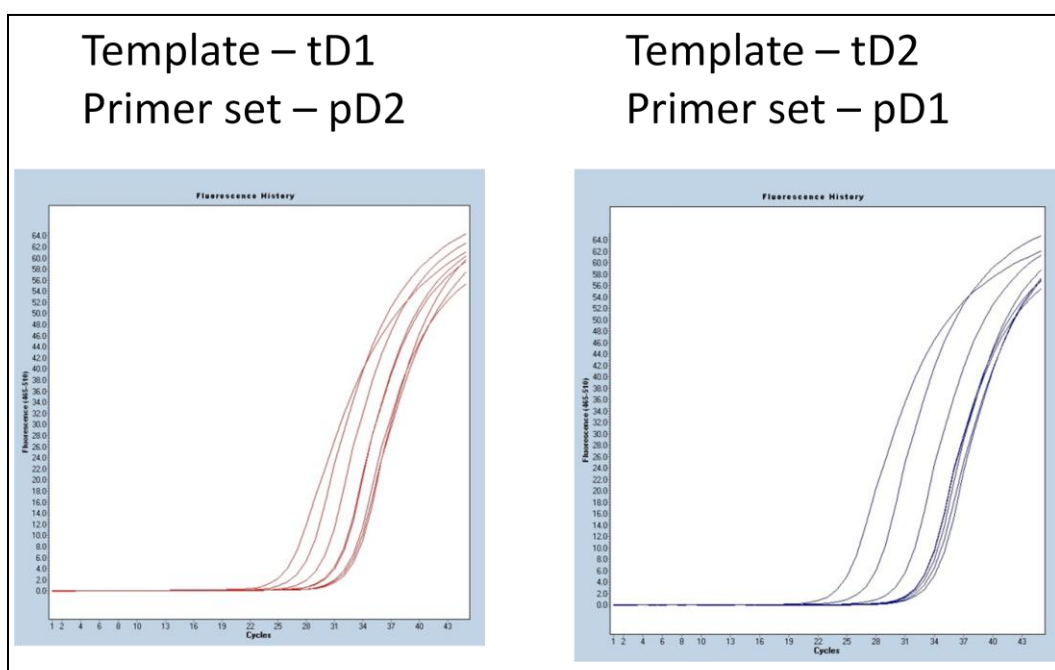

Figure 4. Specificity of primer sets pD1 and pD2. The same serial template dilutions as the ones used in figure 3 are used here. The maximum amount of mismatched template undetectable by the two SNP-primer sets is 0.01 ng or less, which corresponds to 4,400 gDNA copies or less.

Selectivity characterizes the ability of SNP-specific primer sets to respond to the simultaneous presence of matching and mismatching templates. Selectivity is coupled to specificity; however, in practice, it is defined via the ratio of the frequency of the matching and mismatching templates. Alternatively, selectivity is expressed as the percentage of detectable matching templates. Here selectivity is measured as the ratio of matching and mismatching templates. The measured selectivity for the two SNP-specific primer sets shown in figure 5 is at least (1:1000). This means that one copy of the matching template is detected in the presence of 1000 copies of the mismatching template. From figure 5, the selectivity of primer set pD1 is 1:1000 and the selectivity of primer set pD2 is 1:4000. The efficiency of the reactions within the range of selective template detection is 110% for primer set pD1 and 102.8% for primer set pD2.
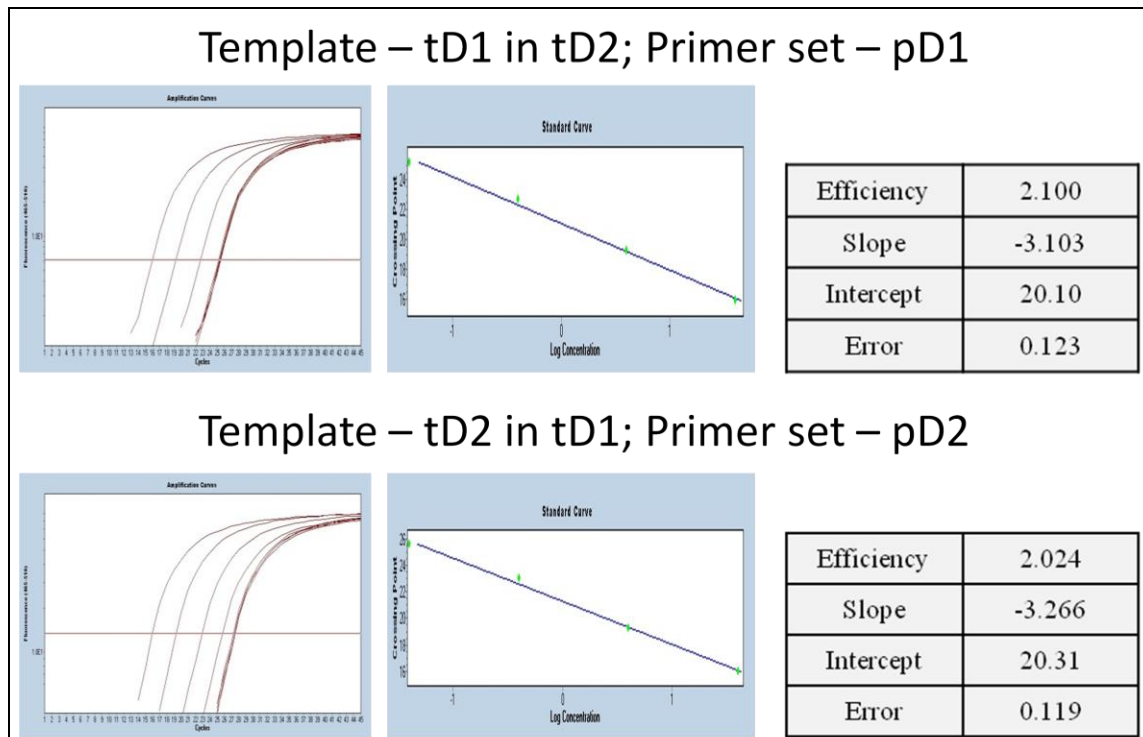
Figure 5. Measuring selectivity of SNP-specific primer sets pD1 and pD2. Each PCR reaction contains 10 ng total of gDNA; however, the matching template from either Detrick-1 or Detrick-2 is different. In the top series, the following ratios of Detrick-1:Detrick-2 (w:w) templates are used: (1:0), (1:10), (1:100), (1:1,000), (1:10,000), (1:100,000), and (1:1,000,000). In the bottom series, the same ratios are used for the Detrick-2:Detrick-1 (v:v) templates.

4.5 Analysis of Competition/Emerging Experiments

The application of the relative quantification PCR method for analysis of competition/emerging experiments is illustrated using a hypothetical emerging experiment, where Detrick-1 emerges in a mixed culture with Detrick-2 or vice versa. The strain with the smaller initial frequency is considered to be the "emerging" strain. When Detrick-2 is the "emerging" strain the experiment is labeled as "Detrick-1 elimination," because by convention Detrick-2 is the y strain for the purposes of using equation 1. One unit of hypothetical generation time is defined as the time necessary for the doubling of the emerging strain. At generation time t_0, the frequency of the emerging strain is set equal to 0.001. Using these conditions, the calculated theoretical selection coefficient ($s1$) is equal to 0.693.

The mixed cultures shown in figure 6 are constructed to mimic the conditions for strain emergence in mixed cultures. OD measurements were used during the construction of the genotypically mixed cultures used in the experiments. gDNA was isolated from the mixed cultures and the total DNA concentration was adjusted to 10 ng per reaction using the internal-control primer set p21115. The C_T values for the reactions using the matching SNP-specific primer sets were measured and placed together with the C_T values for the serial dilution

experiments in figure 5. The species mixing ratios (see legend of figure 6) were used to determine the position of the measured C_T values on the x-axes. The combined data sets are shown as left graphs in figure 6. The graphs show that the C_T values measured for the mixed cultures are in very good agreement with their counterparts measured for the template dilution series. Therefore, the efficiencies from figure 5 are used for the calculation of $\ln\left(\frac{P_{x(t)}}{P_{y(t)}}\right)$ in the right graphs in figure 6. The selection coefficients calculated from the slopes of the dependence of $\ln\left(\frac{P_{x(t)}}{P_{y(t)}}\right)$ on the hypothetical generation time shown in figure 6 were equal to 0.611 for the hypothetical emergence of Detrick-1 and 0.606 for the hypothetical emergence of Detrick-2. These values are close to the theoretical selection coefficient equal to 0.694 calculated above. This demonstrates the utility of the proposed relative PCR quantification approach for analysis of competition experiments.
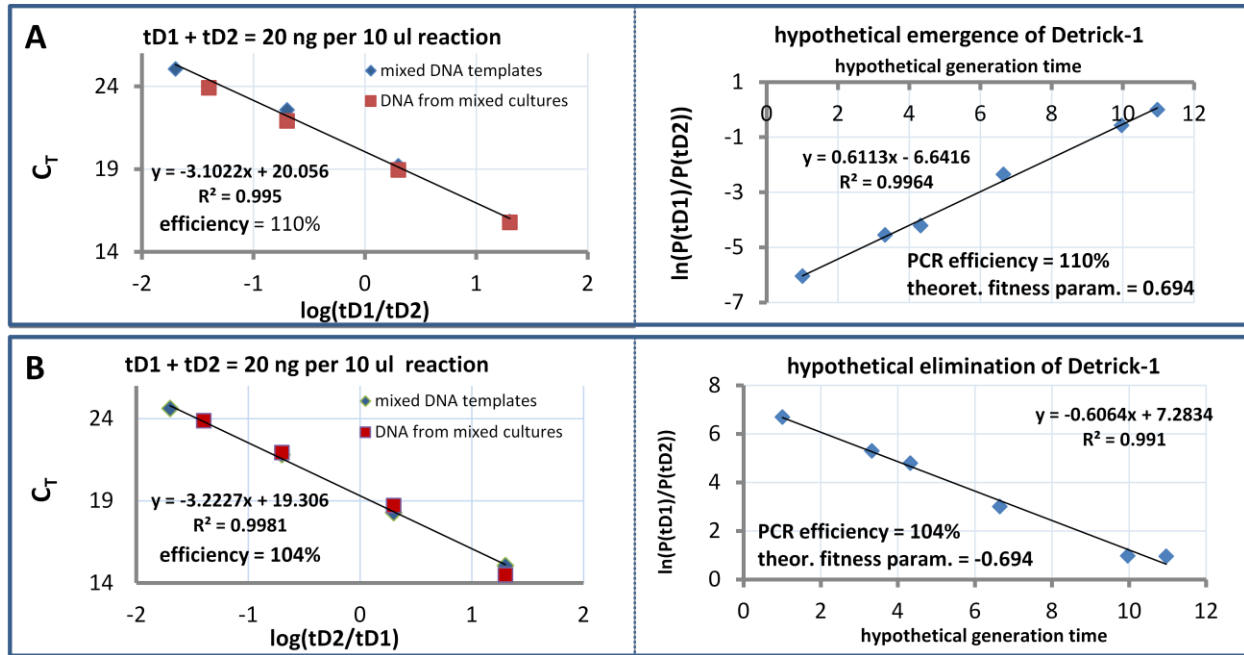

Figure 6. Hypothetical competition experiments for the emergence of (a) Detrrick-1 and (b) Detrick-2 in mixed cultures. Left graphs compare the measured C_T values from mixed cultures (red data points) with the C_T values from the serial dilution experiments in figure 5 (blue data points). The right graphs show the calculated $\ln\left(\frac{P_{x(t)}}{P_{y(t)}}\right)$ using the measured C_T values for mixed cultures (equation 1) vs. hypothetical generation time. The ratios of Detrick-1 and Detrick-2 cells used in the calculation of $\ln\left(\frac{P_{x(t)}}{P_{y(t)}}\right)$ are top (Detrick-1:Detrick-2) = (0.002), (0.01), (0.02), (0.1), (0.5) and (1.0); bottom (Detrick-2:Detrick-1) = (0.002), (0.01), (0.02), (0.1), (0.5) and (1.0).   The slope of this dependence is the rate of change in frequency or the selection coefficient (for non-interacting unlimited cultures). The calculated selection coefficient from the PCR data for emerging Detrick-1 is equal to 0.611 and for Detrick-2 it is equal to 0.606. The theoretical fitness parameter (*51*) is 0.694.

5. Conclusions

A novel approach for analysis of competition experiments based on the use of SNP-specific primer sets and the relative PCR quantification method is developed. The utility of the approach is validated using the BG species Detrick-2 and Detrick-1. These species are suitable model system for single-locus haploid selection experiments because they are isogenic except for a single (C:T) SNP corresponding to the H101R mutation of Spo0F (*16*). The developed assay detects the (C:T) SNP of Spo0F with (1:1000) selectivity, which is sufficient for analysis of competition experiments, or the detection of the Army simulant strain Detrick-2 or its progenitor species in environmental samples.

6. References

1. Zhelev, D. V.; Hunt, M.; Le, A.; Dupuis, C.; Ren, S.; Gibbons, H. et al. Effect on Strain Fitness of the H101R Mutation of Spo0F of Bacillus atrophaeus var globigii, 2012.

2. Hill, V. R.; Polaczyk, A. L.; Hahn, D.; Narayanan, J.; Cromeans, T. L.; Roberts, J. M. et al. Development of a Rapid Method for Simultaneous Recovery of Diverse Microbes in Drinking Water by Ultrafiltration with Sodium Polyphosphate and Surfactants. *Applied and Environmental Microbiology* **2005**, *71* (11), 6878–84.

3. Hodgson, C. J.; Perkins, J.; Labadz, J. C. Evaluation of Biotracers to Monitor Effluent Retention Time in Constructed Wetlands. *Letters in Applied Microbiology* **2003**, *36* (6), 362–71.

4. Hodgson, C. J.; Perkins, J.; Labadz, J. C. The Use of Microbial Tracers to Monitor Seasonal Variations in Effluent Retention in a Constructed Wetland. *Water Research* **2004**, *38* (18), 3833–44.

5. Horan, N. J.; Parr, J.; Naylor, P. J. Evaluation of Tracers for the Determination of the Mixing Characteristics of Activated Sludge Reactors. *Environmental Technology* **1991**, *12* (7), 603–08.

6. Francis, C. A.; Lockley, A. C.; Sartory, D. P.; Watkins, J. A Simple Modified Membrane Filtration Medium For The Enumeration Of Aerobic Spore-Bearing Bacilli In Water. *Water Research* **2001**, *35* (15), 3758–61.

7. Johnson-White, B.; Lin, B.; Ligler, F. S. Combination of Immunosensor Detection with Viability Testing and Confirmation Using the Polymerase Chain Reaction and Culture. *Analytical Chemistry* **2006**, *79* (1), 140–46.

8. Jokerst, J. V.; Jacobson, J. W.; Bhagwandin, B. D.; Floriano, P. N.; Christodoulides, N.; McDevitt, J. T. Programmable Nano-Bio-Chip Sensors: Analytical Meets Clinical. *Analytical Chemistry* **2010**, *82* (5), 1571–79.

9. Kaman, W. E.; Hulst, A. G.; van Alphen, P.T.W.; Roffel, S.; van der Schans, M. J.; Merkel, T. et al. Peptide-Based Fluorescence Resonance Energy Transfer Protease Substrates for the Detection and Diagnosis of Bacillus Species. *Analytical Chemistry* **2011**, *83* (7), 2511–17.

10. Li, D.; Truong, T. V.; Bills, T. M.; Holt, B. C.; VanDerwerken, D. N.; Williams, J. R. et al. GC/MS Method for Positive Detection of Bacillus anthracis Endospores. *Analytical Chemistry* **2011**, *84* (3), 1637–44.

11. Mwilu, S. K.; Aluoch, A. O.; Miller, S.; Wong, P.; Sadik, O. A.; Fatah, A. A. et al. Identification and Quantitation of Bacillus globigii Using Metal Enhanced Electrochemical Detection and Capillary Biosensor. *Analytical Chemistry* **2009**, *81* (18), 7561–70.

12. Saito, S.; Massie, T. L.; Maeda, T.; Nakazumi, H.; Colyer, C. L. On-Column Labeling of Gram-Positive Bacteria with a Boronic Acid Functionalized Squarylium Cyanine Dye for Analysis by Polymer-Enhanced Capillary Transient Isotachophoresis. *Analytical Chemistry* **2012**, *84* (5), 2452–58.

13. Ehrhardt, C. J.; Chu, V.; Brown, T.; Simmons, T. L.; Swan, B. K.; Bannan, J. et al. Use of Fatty Acid Methyl Ester Profiles for Discrimination of Bacillus Cereus T-Strain Spores Grown on Different Media. *Applied and Environmental Microbiology* **2010**, 76 (6), 1902–12.

14. Cliff, J. B.; Jarman, K. H.; Valentine, N. B.; Golledge, S. L.; Gaspar, D. J.; Wunschel, D. S. et al. Differentiation of Spores of Bacillus subtilis Grown in Different Media by Elemental Characterization Using Time-of-Flight Secondary Ion Mass Spectrometry. *Applied and Environmental Microbiology* **2005**, *71* (11), 6524–30.

15. Pellegrino, P. M.; Fell, N. F.; Rosen, D. L.; Gillespie, J. B. Bacterial Endospore Detection Using Terbium Dipicolinate Photoluminescence in the Presence of Chemical and Biological Materials. *Analytical Chemistry* **1998**, *70* (9), 1755–60.

16. Gibbons, H. S.; Broomall, S. M.; McNew, L. A.; Daligault, H.; Chapman, C.; Bruce, D. et al. Genomic Signatures of Strain Selection and Enhancement in *Bacillus atrophaeus var. globigii*, a Historical Biowarfare Simulant. *Plos One* **2011**, *6* (3), e17836.

17. Stephens, C. Bacterial Sporulation: A Question of Commitment? *Current biology: CB* **1998**, *8* (2), R45–R48.

18. Schultz, D.; Wolynes, P. G.; Jacob, E. B.; Onuchic, J. N. Deciding Fate in Adverse Times: Sporulation and Competence in Bacillus Subtilis. *Proceedings of the National Academy of Sciences* **2009**, *106* (50), 21027–34.

19. Burbulys, D.; Trach, K. A.; Hoch, J. A. Initiation of sporulation in B. subtilis is controlled by a multicomponent phosphorelay. *Cell* **1991**, *64* (3), 545–52.

20. Tzeng, Y-L; Zhou, X. Z.; Hoch, J. A. Phosphorylation of the Spo0B Response Regulator Phosphotransferase of the Phosphorelay Initiating Development inBacillus subtilis. *Journal of Biological Chemistry* **1998**, *273* (37), 23849–55.

21. Hoch, J. A. Two-component and Phosphorelay Signal Transduction. *Current Opinion in Microbiology* **2000**, *3* (2), 165–70.

22. Molle, V.; Fujita, M.; Jensen, S. T.; Eichenberger, P.; González-Pastor, J. E.; Liu, J. S. et al. The Spo0A Regulon of Bacillus Subtilis. *Molecular Microbiology* **2003**, *50* (5), 1683–701.

23. Jiang, M.; Tzeng, Y-L; Feher, V. A.; Perego, M.; Hoch, J. A. Alanine Mutants of the Spo0F Response Regulator Modifying Specificity for Sensor Kinases in Sporulation Initiation. *Molecular Microbiology* **1999**, *33* (2), 389–95.

24. McLaughlin, P. D.; Bobay, B. G.; Regel, E. J.; Thompson, R. J.; Hoch, J.A.; Cavanagh, J. Predominantly Buried Residues in the Response Regulator Spo0F Influence Specific Sensor Kinase Recognition. *FEBS Letters* **2007**, *581* (7), 1425–29.

25. Hayward, A. E.; Marchetta, J. A.; Hutton, R. S. Strain Variation as a Factor in the Sporulating Properties of the So-called Bacillus globigii. *J. Bacteriol.* **1946**, *52* (1), 51–54.

26. Liberman, U.; Van Cleve, J.; Feldman, M. W. On the Evolution of Mutation in Changing Environments: Recombination and Phenotypic Switching. *Genetics* **2011**, *187* (3), 837–51.

27. Bjedov, I.; Tenaillon, O.; Gérard, B.; Souza, V.; Denamur, E.; Radman, M. et al. Stress-Induced Mutagenesis in Bacteria. *Science* **2003**, *300* (5624), 1404–09.

28. Kurland, C. G.; Canback, B.; Berg, O. G. Horizontal Gene Transfer: A Critical View. *Proceedings of the National Academy of Sciences* **2003**, *100* (17), 9658–62.

29. Avery, S. V. Microbial Cell Individuality and the Underlying Sources of Heterogeneity. *Nat Rev Micro* **2006**, *4* (8), 577–87.

30. Ben-Jacob, E.; Levine, H. Self-engineering Capabilities of Bacteria. *Journal of The Royal Society Interface* **2006**, *3* (6), 197–214.

31. Leibler, S.; Kussell, E. Individual Histories and Selection in Heterogeneous Populations. *Proceedings of the National Academy of Sciences* **2010**, *107* (29), 13183–88.

32. Barrick, J. E.; Yu, D. S.; Yoon, S. H.; Jeong, H.; Oh, T. K.; Schneider, D. et al. Genome Evolution and Adaptation in a Long-term Experiment with Escherichia Coli. *Nature* **2009**, *461* (7268), 1243–47.

33. Herring, C. D.; Raghunathan, A.; Honisch, C.; Patel, T.; Applebee, M. K.; Joyce, A. R. et al. Comparative Genome Sequencing of Escherichia Coli Allows Observation of Bacterial Evolution on a Laboratory Timescale. *Nat Genet* **2006**, *38* (12), 1406–12.

34. Zdziarski, J.; Brzuszkiewicz, E.; Wullt, B.; Liesegang, H.; Biran, D.; Voigt, B. et al. Host Imprints on Bacterial Genomes—Rapid, Divergent Evolution in Individual Patients. *PLoS Pathog* **2010**, *6* (8), e1001078.

35. MacLean, R. C.; Perron, G. G.; Gardner, A. Diminishing Returns From Beneficial Mutations and Pervasive Epistasis Shape the Fitness Landscape for Rifampicin Resistance in Pseudomonas aeruginosa. *Genetics* **2010**, *186* (4), 1345–54.

36. Brown, C. T.; Fishwick, L. K.; Chokshi, B. M.; Cuff, M. A.; Jackson, J. M.; Oglesby, T. et al. Whole-Genome Sequencing and Phenotypic Analysis of Bacillus Subtilis Mutants Following Evolution under Conditions of Relaxed Selection for Sporulation. *Applied and Environmental Microbiology* **2011**, *77* (19), 6867–77.

37. Nicholson, W. L.; Fajardo-Cavazos, P.; Fedenko, J.; Ortíz-Lugo, J. L.; Rivas-Castillo, A.; Waters, S. M. et al. Exploring the Low-Pressure Growth Limit: Evolution of Bacillus subtilis in the Laboratory to Enhanced Growth at 5 Kilopascals. *Applied and Environmental Microbiology* **2010**, *76* (22), 7559–65.

38. Sampath, R.; Russell, K. L.; Massire, C.; Eshoo, M. W.; Harpin, V.; Blyn, L. B. et al. Global Surveillance of Emerging Influenza Virus Genotypes by Mass Spectrometry. *Plos One* **2007**, *2* (5), e489.

39. McCaughan, F.; Dear, P. H. Single-molecule Genomics. *The Journal of Pathology* **2010**, *220* (2), 297–306.

40. Hindson, B. J.; Ness, K.D.; Masquelier, D. A.; Belgrader, P.; Heredia, N. J.; Makarewicz, A. J. et al. High-Throughput Droplet Digital PCR System for Absolute Quantitation of DNA Copy Number. *Analytical Chemistry* **2011**, *83* (22), 8604–10.

41. Dominguez, P. L.; Kolodney, M. S. Wild-type Blocking Polymerase Chain Reaction for Detection of Single Nucleotide Minority Mutations from Clinical Specimens. *Oncogene* **2005**, *24* (45), 6830–34.

42. Sheludchenko, M. S.; Huygens, F.; Hargreaves, M. H. Highly Discriminatory Single-Nucleotide Polymorphism Interrogation of Escherichia coli by Use of Allele-Specific Real-Time PCR and eBURST Analysis. *Applied and Environmental Microbiology* **2010**, *76* (13), 4337–45.

43. Morlan, J.; Baker, J.; Sinicropi, D. Mutation Detection by Real-Time PCR: A Simple, Robust and Highly Selective Method. *Plos One* **2009**, *4* (2), e4584.

44. Ugozzoli, L. A.; Latorra, D.; Pucket, R.; Arar, K.; Hamby, K. Real-time Genotyping with Oligonucleotide Probes Containing Locked Nucleic Acids. *Analytical Biochemistry* **2004**, *324* (1), 143–52.

45. Cheng, J.; Zhang, Y.; Li, Q. Real-time PCR Genotyping Using Displacing Probes. *Nucleic Acids Research* **2004**, *32* (7), e61.

46. Kutyavin, I. V.; Afonina, I. A.; Mills, A.; Gorn, V. V.; Lukhtanov, E. A.; Belousov, E. S. et al. 3′-Minor Groove Binder-DNA Probes Increase Sequence Specificity at PCR Extension Temperatures. *Nucleic Acids Research* **2000**, *28* (2), 655–61.

47. Livak, K. J. Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2-\Delta\Delta CT$ Method. *Methods* **2001**, *25* (4), 402–08.

48. Vulic. M.; Kolter, R. Evolutionary Cheating in Escherichia coli Stationary Phase Cultures. *Genetics* **2001**, *158* (2), 519–26.

49. Liggett, R. W.; Koffler, H. Corn Steep Liquor in Microbiology. *Bacteriol. Rev.* **1948**, *12*, 297–311.

50. Roth, N. G.; Lively, D. H.; Hodge, H. M. Influence of Oxygen Uptake and Age of Culture on Sporulation of Bacillus Antracis and Bacillus Globigii. *J. Bacteriol.* **1955**, *69*, 455–59.

51. Chevin, L-M. On Measuring Selection in Experimental Evolution. *Biology Letters* **2011**, *7* (2), 210–13.

52. Lenski, R. E.; Rose, M. R.; Simpson, S. C.; Tadler, S. C. Long-Term Experimental Evolution in Escherichia coli. I. Adaptation and Divergence During 2,000 Generations. *Am. Nat.* **1991**, *138* (6), 1315–41.

List of Symbols, Abbreviations, and Acronyms

BG	*Bacillus atrophaeus var globigii*
C_T	cycle-threshold
CSL	corn steep liquor
Detrick-1	low spore yield variant of Bacillus atrophaeus var globigii
Detrick-2	high spore yield variant of Bacillus atrophaeus var globigii
DMSO	dimethyl sulfoxide
E	efficiency of real-time PCR reaction
gDNA	genomic DNA
LB	lysogeny broth
OD	optical density
$P_x(t)$	frequency of a species in mixed cultures
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pD1, pD2 and p21115	primer sets
PI	propidium iodide
qPCR	quantitative PCR
SNP	single nucleotide polymorphism
Spo0A	transcription factor (response) regulator
Spo0B	phosphotransferase
Spo0F	two component response regulator
t	time measured in generations
tD1 and tD2	genomic DNA templates